Immunology, 1971, **20**, 755.

Cellular Immunity in Experimental Glomerulonephritis of Rats*

I. DELAYED HYPERSENSITIVITY AND LYMPHOCYTE STIMULATION STUDIES WITH RENAL TUBULAR ANTIGENS

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(*Received 8th April* 1970)

Summary. Experimental glomerulonephritis (EGN) was produced in Sprague-Dawley rats with a single intradermal injection of a renal tubular fraction called Fx1A. Presence of disease was confirmed by proteinuria and by light and electron microscopic changes. This antigen and also the non-nephritogenic antigen, Fx1B, were used to study cellular immunity in nephritic and three groups of control rats.

64 per cent (9/14) of the Fx1A immunized rats gave delayed hypersensitivity skin tests to Fx1A. The difference between the experimental and the three control groups was highly significant.

Peripheral blood lymphocyte culture responses to Fx1A, Fx1B, phytohaemagglutinin-P, were studied in the nephritic and control groups. 77 per cent (10/13) of Fx1A immunized animals showed stimulation of their lymphocyte cultures with Fx1A. None of the control groups showed any significant stimulation.

These experiments have demonstrated cellular immunity in EGN and support the possibility that these mechanisms may play a role in the production of this disease.

INTRODUCTION

In 1959, Heymann produced a chronic glomerulonephritis in rats with multiple injections of crude kidney homogenate emulsified with Freund's complete adjuvant (Heymann, Hackel, Harwood, Wilson and Hunter, 1959). The renal antigen responsible for this type of experimental glomerulonephritis (EGN), has been localized in a saline insoluble renal tubular fraction called Fx1A (Edgington, Glassock and Dixon, 1967). By immunofluorescence, the antigen in Fx1A responsible for the nephritis was located in the apical portion of the epithelial cells of the renal proximal convoluted tubules (Edgington *et al.*, 1967). Recently, this antigen has been chemically isolated from Fx1A, designated RTEα5 and has properties of a lipoprotein (Edgington, Glassock and Dixon, 1968). Immunization of rats with this non-glomerular renal antigen results in a glomerulonephritis which is associated temporally with the deposition on the glomerular basement membrane of antigen–antibody complexes containing RTEα5, γ2-globulin and β1c-globulin (Glassock,

* Supported by United States Public Health Service Grant AM 09577.
† Supported by United States Public Health Service Grant FR-5408.
‡ Work performed during Senior Investigatorship of the Arthritis Foundation.

Edgington, Watson and Dixon, 1968). Previous evidence had suggested that cellular immune mechanisms may play a role in the production of this disease (Hess, Ashworth and Ziff, 1962; Heymann, Hunter, Hackel and Cuppage, 1962; Holm, 1966; Grupe, 1968). The experiments here described demonstrate cellular immunity in EGN utilizing classic delayed hypersensitivity skin tests and antigen induced blast transformation of peripheral blood lymphocytes.

MATERIALS AND METHODS

Animals

Adult female Sprague-Dawley rats (Sprague-Dawley, Inc., Madison, Wisconsin) weighing 150–200 g were used.

Urinary protein measurements

24-hour urinary proteins were determined quantitatively by the Shevky-Stafford method (Shevky and Stafford, 1923).

The normal range in this laboratory is 0·34 mg to 6·0 mg/24 hours depending on the age of the rat. Proteinuria was considered to be present if more than 10 mg/24 hours were consistently excreted.

Preparation of renal antigens

The method of Edgington and associates was followed with sterile conditions maintained during the entire preparation (Edgington *et al.*, 1968). In brief, the renal cortices obtained from saline flushed rat kidneys were forced through a 150 mesh stainless steel sieve. The resulting suspension was centrifuged at 400 ***g*** to remove the glomeruli. The supernate was centrifuged at 78,640 ***g*** to separate two renal tubular fractions, the supernatant fluid which contained Fx1B, a non-nephritogenic renal tubular fraction, and the pellet which contained Fx1A, a renal tubular fraction which includes the nephritogenic antigen. Both fractions were lyophilized and their protein contents determined by the Lowry method (Lowry, Rosebrough, Farr and Randall, 1957).

Plan of the experiment

Four groups of rats were studied, one experimental and three control groups. All antigens were administered emulsified in Freund's complete adjuvant (CFA) (Difco Laboratories, Detroit) which contained 4 mg/ml *Mycobacterium tuberculosis* H37Ra in a volume of 0·2 ml into one hind foot pad intradermally. The experimental group was immunized with 10 mg Fx1A in CFA, the first control group with 10 mg Fx1B in CFA, the second control group with CFA alone and the third control group remained uninjected. 24-hour urinary protein excretion was determined weekly on each rat. When rats in the experimental group (Fx1A injected) developed proteinuria which remained constant for at least 3 weeks, skin tests with Fx1A, Fx1B and PPD were performed (usually at about the 20th post-immunization week). One week after the skin tests, lymphocyte cultures were performed with blood obtained by cardiac puncture, to test the same antigens. Fx1A contained 60 per cent protein and the Fx1B, 40 per cent protein.

Skin tests

The concentration of antigens and control solutions used in skin testing were as follows: Fx1A, 1 μg/ml and 0·1 μg/ml; Fx1B, 1 μg/ml; 0·9% sodium chloride; sodium

desoxycholate, 0·0001 per cent. Sodium desoxycholate is a detergent which was required for solubilization of Fx1A in aqueous solution (Edgington *et al.*, 1968).

0·1 ml of the various antigens and control solutions were injected intradermally into the shaved flanks of the rats. The skin test sites were inspected for evidence of induration, erythema and necrosis at 4, 24, 48 and 72 hours. Criteria for a positive skin test for delayed hypersensitivity included the presence of 5 mm or greater induration 24 hours after injection and the presence of necrosis. These criteria were based on our observation that injection of sodium desoxycholate 0·0001 per cent control solution, could produce up to 4 mm induration 24 hours after injection.

Representative skin test sites were examined histologically.

Peripheral blood lymphocyte cultures

The method used is a modification of Wilson's (Wilson, 1967). 8 ml of blood obtained by cardiac puncture were drawn into a syringe containing 1 ml of 10 per cent dextran and 1 ml of 5 per cent sodium citrate. After sedimentation, the leucocyte rich plasma was removed and the leucocytes washed three times with Hanks's balanced salt solution (Grand Island Biological Co., Grand Island, New York). The cells were resuspended and the concentration adjusted to 1×10^6 mononuclear cells/ml with Earle's balanced salt solution (Grand Island Biological Co., Grand Island, New York) which contained twice the usual amount of amino acids and vitamins (Flow Laboratories, Rockville, Maryland) and 15 per cent AB positive plasma. 1-ml aliquots of the cell suspension were dispensed into glass disposable culture tubes (Becton-Dickinson, Rutherford, New Jersey) with Morton stainless steel caps (Bellco Biological Glassware and Equipment, Vineland, New Jersey) and incubated upright with the proper antigens or phytohaemagglutinin-P (PHA-P) (Difco Laboratories, Detroit, Michigan) in an atmosphere of 95 per cent moist air and 5 per cent CO_2 at 37°. 16 hours prior to harvesting (which was 3 days of incubation for PHA-P and 5 days for antigens and control cultures), 0·25 μCi of [3H]thymidine (specific activity 11 Ci/mM) (Schwartz Bioresearch, Orangeburg, New York) was added to each culture. At the time of harvest, the cultures were initially chilled and then centrifuged at 1500 rev/min for 10 minutes. After centrifugation, 2 ml of ice cold 5 per cent tricholoroacetic acid were added and the resulting precipitate washed twice more with 1 ml of ice cold 5 per cent tricholoroacetic acid. Digestion of this precipitate was carried out with 0·5 ml of hydroxide of hyamine (10×) at 60° for 20 minutes. This digest was then transferred to glass counting vials containing 2 ml of absolute alcohol and heated at 80° for 90 minutes. Then 17·5 ml of scintillation fluid (0·3 per cent PPO and 0·01 per cent POPOP in toluene) was added and the vials counted in a tri-carb liquid scintillation counter (Packard Instrument Co., Downers Grove, Illinois). Quenching was determined by the channels ratio method and by external standardization. Results of cultures were expressed as:

$$\frac{\text{Counts/min of antigen or mitogen culture minus background}}{\text{Counts/min of control cultures minus background}} = \text{Ratio}$$

The agents used in lymphocyte cultures were as follows: Fx1A, 0·1 μg and 0·01 μg/culture; Fx1B, 0·1 μg/culture; PHA-P, 0·1 ml of 1:1000 solution/culture; sodium desoxycholate, 0·1 ml of 0·0001 per cent/culture All antigen, mitogen and control cultures were done in triplicate. As sodium desoxycholate is toxic to rat peripheral blood lymphocyte cultures, the results of cultures incubated with Fx1A were always compared to control

cultures incubated with 0·0001 per cent sodium desoxycholate, a concentration which produces minimal or no toxic effects.

Histology

Kidneys were fixed immediately in either buffered formalin or in Bouin's solution. Skin biopsies were fixed in formalin. The sections were stained with haematoxylin and eosin and by the periodic acid Schiff method. For electron microscopy, the blocks were initially fixed in cold phosphate buffer and 4 per cent glutaraldehyde for 1–4 hours. The blocks were then washed in phosphate buffer for 1–3 hours, fixed in 1 per cent osmium tetroxide for 1 hour, dehydrated in ethanol and embedded in Epon or Maraglas. Sections were stained with uranyl acetate and lead citrate and examined with an RCA-EMU-3F electron microscope.

RESULTS

DEVELOPMENT OF PROTEINURIA

In the FxlA immunized rats, proteinuria first appeared 8 weeks after immunization with a mean value of 13 mg/24 hours for the group. Excretion of protein then progressively increased to 50 mg/24 hours mean value at 12 weeks and 63 mg/24 hours mean value at 16 weeks and then achieved relative stability. Proteinuria was not present in any of the control group rats except for an occasional transient elevation. Ascites or oedema was not observed, in contrast to those injected with kidney antigens intraperitoneally in previous experiments (Hess *et al.*, 1962).

HISTOLOGY

Kidneys: (a) *light microscopy*

The kidney sections in the experimental group (FxlA injected) all showed similar changes (Fig. 1). The glomeruli were normal in size and the cellular content was either normal or slightly increased. Mesangial stalks were prominent and exhibited a slightly increased nuclear content; cytoplasm was abundant and deeply eosinophilic. Glomerular epithelium was markedly swollen and filled the urinary spaces; cytoplasm was abundant, pale staining, and the nuclei large, ovoid, and vesicular. Endothelial cells appeared normal. Basement membranes were diffusely but minimally thickened; at higher magnification multiple delicate spikes projected toward the epithelial cytoplasm from the outer surface of the basement membrane. These were especially prominent over the peripheral capillary loops. In the proximal tubules there were rare PAS-positive hyaline droplets; mild haemosiderosis was evident in the distal tubules. The vessels and interstitium were unremarkable.

(b) *Electron microscopy*

Basement membrane. The endothelial surface of the basement membrane was smooth. The epithelial surface, however, was markedly irregular with a saw-tooth appearance due to multiple deposits of electron dense material varying in size. Some deposits were clearly encased by the basement membrane and protruded into fused foot processes or bulged between processes. Similar deposits were also numerous in the foot processes, trabeculae

of the epithelial cells and lamina rara externa (Fig. 2). In those instances in which a heavy deposit in the foot process coincided with one in the basement membrane, both the cell membrane and lamina rara externa were lost and a continuous sheet of dense deposits appeared to pass from one to the other. Also in the basement membrane were various sized floccular foci of basement membrane 'fall-out'; the 'fall-out' area was partly filled with granular material. In some cases, dense deposits were extensive in the basement membranes covering the mesangial stalk and in the overlying epithelium (Fig. 3).

Epithelial cells. In the epithelium, swollen trabecular cytoplasmic projections extended prominently out into and filled the urinary spaces. Many Golgi complex fragments clustered around otherwise unremarkable nuclei; the fragments were mainly situated on

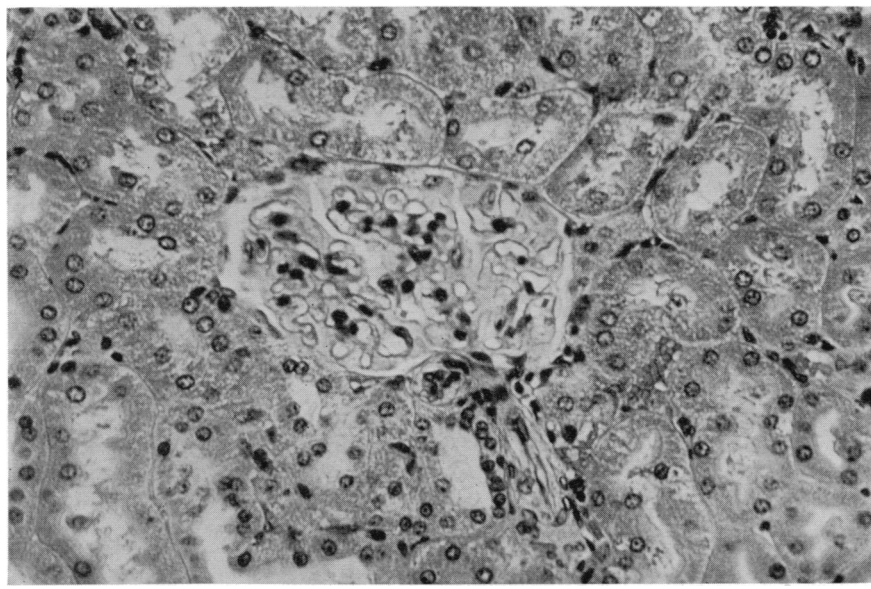

FIG. 1. Light photomicrograph of a PAS stained kidney of an Fx1A immunized rat with proteinuria. The epithelial cells of the glomerular capillary tuft are prominent because of the swollen cytoplasm partly filling the urinary space. The basement membrane is diffusely thickened (× 180).

the basement membrane side of the cell. In a few cases, many membrane-limited vacuoles filled with an osmophilic substance of varied density (cytolysomes) were seen in the cytoplasm (Fig. 3). Areas in which foot processes remained delicate were scattered but in the main areas these were fused and contained dense deposits.

Mesangial cells. In general, the mesangial stalk was slightly widened. Peripheral cytoplasm covered by attenuated portions of endothelium frequently extended into the capillary for a short distance.

Endothelial cells. The capillary lumens in all cases were normally patent but the attenuated portions of the endothelial cytoplasm appeared thickened with more pinocytotic vesicles than normal.

Skin

The Fx1A injected sites that were considered positive showed varying degrees of necrosis involving the epidermis, dermis, subcutaneous fat and underlying muscle with

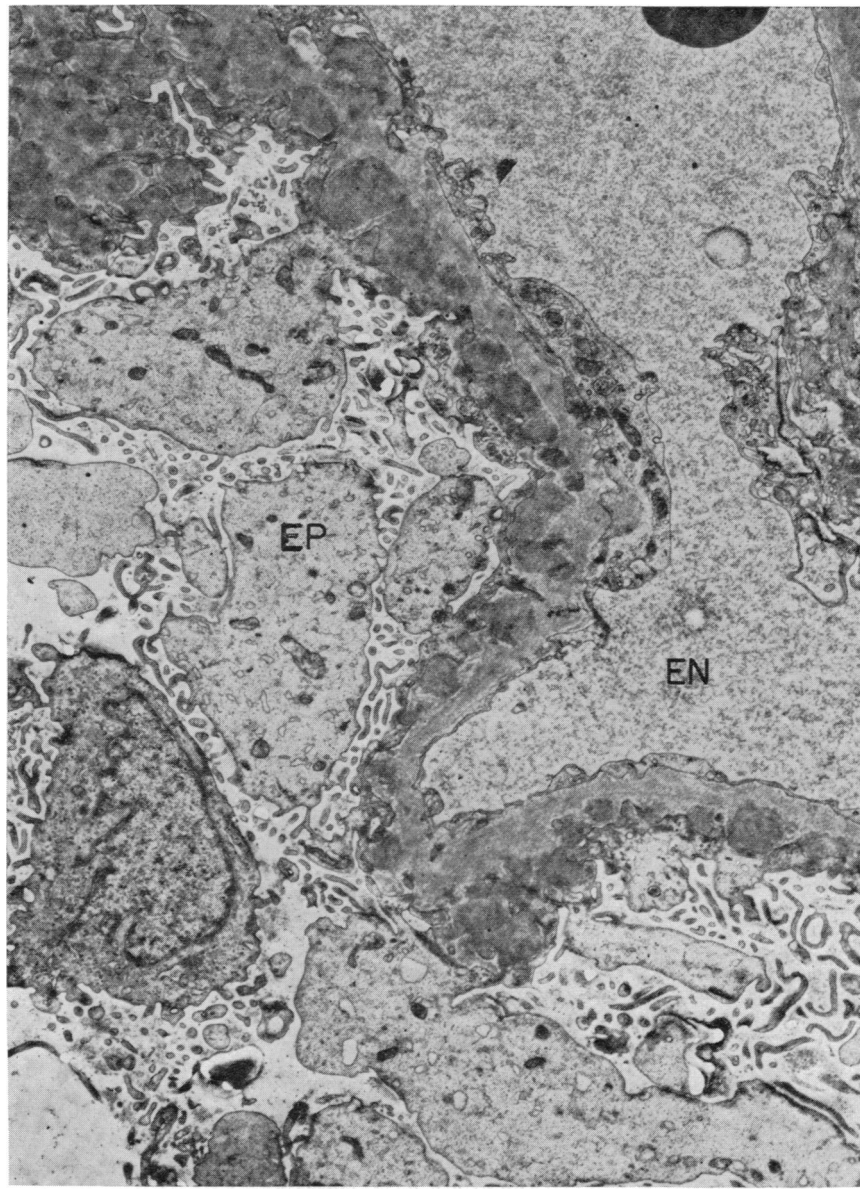

FIG. 2. Electron micrograph of an Fx1A immunized rat with proteinuria. Irregular sawtooth appearance of outer surface of basement membrane due to electron dense deposits within basement membrane and between foot processes and lamina rara externa. Foot processes overlying lesions are fused and contain deposits. Swollen trabecular cytoplasmic projections of epithelial cells fill urinary space (×3900). Ep = epithelial cell, EN = endothelial cell.

heavy exudation mainly of lymphocytes and macrophages around small vessels. Neutrophils were sparse and capillary proliferation was prominent. Arteries and veins occasionally exhibited focal fibrinoid necrosis accompanied by heavy perivascular infiltration with mononuclear cells (Fig. 4).

SKIN TESTS

The results obtained by skin testing each group of rats with Fx1A (1 μg/ml) are shown in Table 1. 64 per cent (9/14) of the Fx1A immunized rats had a positive skin test but only one of the control rats showed a response. This was a CFA immunized animal. The difference between the experimental and the three control groups is significant ($P \ll 0{\cdot}05$ by the Kruskal-Wallis test, a one way analysis of variance test for non-parametric data (Sokal and Rohlf, 1969)). The number of positive reactions might have been higher

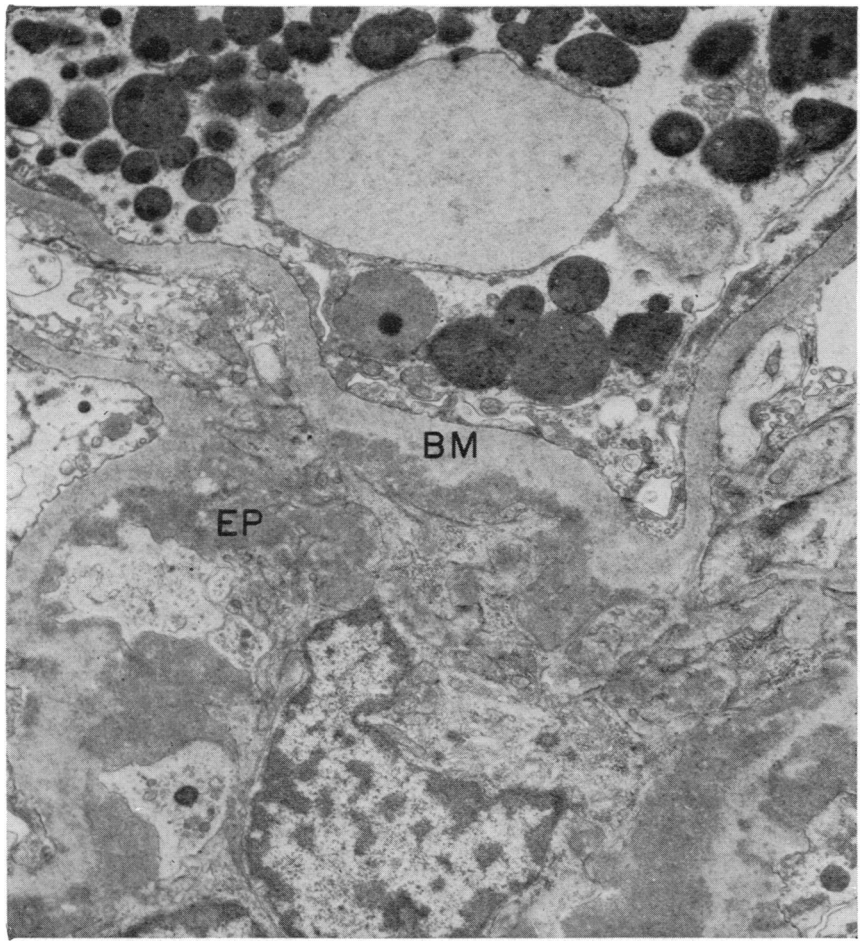

FIG. 3. Electron micrograph of another Fx1A immunized rat. Dense deposits are extensive in basement membrane network of mesangial stalk. Epithelial cell contains membrane-limited vacuoles filled with osmophilic substance of varied density (cytolysosomes) (×4900). BM = basement membrane, EP = epithelial cell.

if a larger testing dose had been employed. Indeed, tests in a few animals support this contention. One group of 10 Fx1A immunized animals was retested following lymphocyte cultures using 100 μg Fx1A for the skin test dose. Nine of ten animals responded with a very large area of induration often accompanied by necrosis within 24 hours. After 48 hours, the animals were killed and the skin test sites examined histologically. All were

consistent with delayed hypersensitivity, showing perivascular lymphocytic and histocytic infiltration. Arthus reactions were not observed. Skin tests with Fx1B were uniformly negative in all groups of rats.

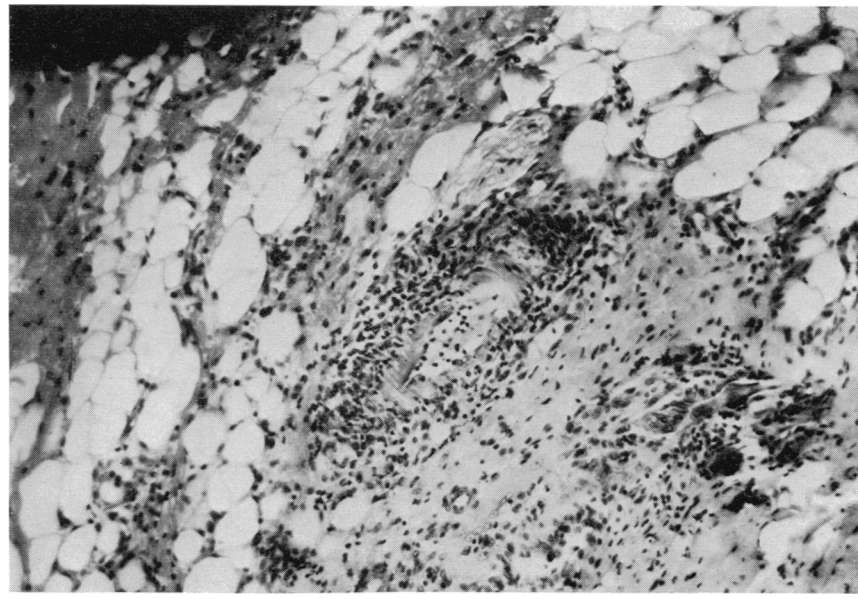

FIG. 4. Light photomicrograph of a haematoxylin and eosin stained skin section from a positive reaction site to intradermal Fx1A in an Fx1A immunized rat. A medium sized artery in the subcutis shows fibrinoid necrosis of the wall and perivascular infiltration by mononuclear cells (×240).

TABLE 1

RESULTS OF INTRADERMAL TESTS WITH Fx1A (1 μg/ml)

Group	No. tested	No. positive*	No. negative
Fx1A immunized rats	14	9†	5
Fx1B immunized rats	10	0	10
Freund's immunized rats	5	1	4
Unimmunized rats	14	0	14

* Criteria Positive Test = > 5 mm induration 24 hours post injection with necrosis.
† $P \ll 0{\cdot}05$ by Kruskal-Wallis test.

TABLE 2

LYMPHOCYTE CULTURE RESPONSE TO Fx1A 0·1 μg/CULTURE

Group	No. tested	No. positive	No. negative
Fx1A immunized rats	13	10*	3
Fx1B immunized rats	8	0	8
Freund's immunized rats	5	0	5
Unimmunized rats	5	0	5

* $P \ll 0{\cdot}05$ by Kruskal-Wallis test.

TABLE 3

MEAN RATIOS OBTAINED IN LYMPHOCYTE CULTURE*

Group	PHA-P 1–1000	Fx1A 0·1 μg/culture	Fx1B 0·1 μg/culture
Fx1A immunized rats	23·5	5·9†	0·8
Fx1B immunized rats	26·0	0·8	0·5
Freund's immunized rats	27·6	1·3	0·7
Unimmunized rats	17·7	0·7	1·0

* $\frac{\text{Counts/min of antigen or mitogen culture minus background}}{\text{Counts/min of control cultures minus background}}$ = Ratio (2 or > significant).

† $P \ll 0{\cdot}05$ by Kruskal-Wallis test.

PERIPHERAL BLOOD LYMPHOCYTE CULTURES

The various groups of rats exhibited clear-cut differences in the response of their peripheral blood lymphocyte cultures with Fx1A, Fx1B and PHA-P. In assessing lymphocyte cultures for evidence of stimulation, a ratio of 2 or more was accepted as indicative of a significant increase over control cultures and was based on our earlier experience with tuberculin-sensitized rats (Litwin, Adams and Hess, unpublished). Table 2 shows the results obtained in the various groups of rats with Fx1A (0·1 μg/culture). 77 per cent (10/13) of rats immunized with Fx1A showed stimulation of their lymphocyte cultures with Fx1A (0·1 μg/culture) but none of the control cultures responded. Table 3 depicts the mean ratios obtained in each group with PHA-P, Fx1A and Fx1B. The response in the Fx1A immunized group to Fx1A (0·1 μg/culture) was highly significant compared to the response with this antigen in the control groups ($P \ll 0{\cdot}05$). The response to Fx1B was

TABLE 4

CORRELATION OF LYMPHOCYTE CULTURE RESULTS, SKIN TESTS AND URINARY PROTEIN IN Fx1A IMMUNIZED RATS

	Lymphocyte Culture							
	PHA-P 1:1000			Fx1A 0·1 mg/culture			Results of i.d. skin test with Fx1A (1 μg/ml)	Urinary protein (mg per 24 hours)†
Rat No.	PHA-P (counts/min)	Control (counts/min)	Mean* ratio	Fx1A at 1 μg/1·0 (counts/min)	Control (counts/min)	Mean* ratio		
1	16,976	903	18·8	2362	842	2·8	Positive	102
2	20,410	570	35·8	1301	540	2·4	Positive	282
3	608	98	6·2	290	109	2·6	Positive	175
4	1628	69	23·6	94	41	2·3	Positive	83
5	2904	88	33·0	629	68	9·3	Positive	40
6	13,189	758	17·4	2781	375	7·4	Negative	53
7	9835	1366	7·2	2826	785	3·6	Negative	40
8	29,900	1625	18·4	1566	198	7·9	Positive	47
9	5569	357	15·6	5578	289	19·3	Positive	27
10	1803	247	7·3	1521	309	4·9	Negative	11
11	4485	115	39·0	38	77	0·5	Positive	30
12	10,474	516	20·3	435	467	0·9	Negative	25
13	33,149	1641	20·2	2280	1422	1·6	Negative	13
14	ND	ND	ND	ND	ND	ND	Positive	52

* = Ratio of 2 or > significant.
† = Mean of two urines.
ND = Technically unsatisfactory, insufficient cells.

not significantly different in the various groups and was always less than a ratio of 2 ($P > 0{\cdot}10$). All groups had excellent though variable responses to PHA-P. However, these differences were not statistically significant ($P \gg 0{\cdot}05$).

Fx1A in a dose of 0·01 μg/culture was tested in a smaller number of rats, because in many experiments the lymphocyte yield was not sufficient to allow testing with the lower dose. Of the nine Fx1A immunized animals tested, the lymphocyte cultures of five showed stimulation but less than that found with the larger test dose of Fx1A, except in one animal.

Table 4 compares the results of the lymphocyte cultures of the Fx1A immunized rats with the skin test results. Three of the rats (No. 11, 12, 13) did not have a significant response to Fx1A in lymphocyte cultures and the skin tests were negative in two of the three. All three of these rats had an excellent PHA response. Three of the ten rats with significant lymphocyte responses had negative skin tests. There was no particular correlation between the intensity of the skin response and the degree of blast transformation in those rats with a significant lymphocyte culture response to Fx1A. All of the rats were proteinuric and had light microscopy changes with varying degrees of involvement.

DISCUSSION

The data presented here provide further evidence that cellular immunity is present in EGN. The skin tests performed with Fx1A in rats with EGN had the rate of development, gross appearance, and histologic characteristics of a cellular immune reaction. The reactions were highly sensitive and very specific, being noted in rats that had been immunized with Fx1A, the renal tubular antigen (except in one instance). The results obtained with peripheral blood lymphocyte cultures support the skin test findings. With Fx1A as test antigen, a similar number of Fx1A immunized rats had a significant increase in blast transformation of their peripheral blood lymphocyte cultures. Similar cultures in the control groups showed negligible stimulation.

Antigen-induced blast transformation of peripheral blood lymphocyte cultures is probably an *in vitro* correlate of delayed hypersensitivity. The reaction has been shown to be carrier rather than hapten specific (Oppenheim, Wolstencroft and Gell, 1967) and requires an antigenic determinant of the same large size needed to elicit delayed skin tests rather than the smaller size needed for an Arthus reaction (Stulbarg and Schlossman, 1968). Antigen-induced blast transformation was not present in guinea-pigs in whom immunization resulted in humoral antibody without delayed hypersensitivity (Mills, 1966). However, this viewpoint has been contested by Benezra, Gery and Davies (1969) who have recently reported that at least in rabbits, antigens eliciting a humoral antibody response or delayed hypersensitivity or both were capable, in each instance, of inducing lymphocyte transformation.

There is additional evidence to support the presence of cellular immunity in this model of experimental glomerulonephritis of rats. The mode of induction of the disease (a single intradermal foot pad injection of the antigen with CFA), passive transfer of the disease by cells rather than serum into tolerant recipients (Hess *et al.*, 1962; Heymann *et al.*, 1962) cytotoxic effects of lymphoid cells on rat kidney cells *in vitro* (Holm, 1966), inhibition of migration of nephritic rat spleen cells by rat kidney extracts (Grupe, 1968) all implicate cellular immune mechanisms. Indeed the experiments that demonstrated passive transfer

of 'autoimmune nephrosis' by cells rather than serum imply that cellular immunity is an active participant in the pathogenesis of EGN.

Recently, the role of antigen–antibody complexes in the causation of EGN has been emphasized. By immunofluorescence, such complexes have been demonstrated in the glomeruli of rats with EGN. These complexes have been shown to contain the renal tubular antigen (RTEα5), γ2-globulin and β1C globulin (Glassock *et al.*, 1968). γ2-Globulin has been eluted from the kidneys of rats with EGN and has been shown by immunofluorescence to react only with the apical portion of the proximal renal tubular epithelial cells, the exact anatomical location of RTEα5 (Grupe and Kaplan, 1969).

The demonstration of antigen–antibody complexes in rats with EGN does not exclude a pathogenetic role for a previously or simultaneously occurring cellular immune reaction. In the model of experimental allergic orchitis in guinea-pigs, Brown, Glynn and Holborow (1967) demonstrated that both cellular immunity and humoral antibody must be present before the disease will appear. The presence of either type of immune response without the other did not result in orchitis. The same situation could exist in EGN.

Obviously, the final answer to the question of the role of cellular immunity in EGN must await future experiments. Such experiments must carefully define the presence, exact role, and the interrelationships of the various humoral antibody responses and cellular immune reactions produced when tolerance is lost to the nephritogenic antigen in EGN.

In the present experiment, Fx1A was the antigen employed. This fraction contains at least two other renal tubular antigens in addition to the nephritogenic one, RTEα5 (Edgington *et al.*, 1968). The persistence of skin tests and lymphocyte transformation responses to Fx1A for at least 20 weeks implies a state of continued immunization and suggests that these responses were made to the nephritogenic antigen contained in Fx1A.

ACKNOWLEDGMENT

The authors wish to express their grateful appreciation to Dr On Ja Kim, Department of Pathology, University of Cincinnati Medical Center, for all her help in the preparation and interpretation of the histological sections.

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